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Influence of dietary inclusion of a wet processed faba bean protein isolate on post-smolt Atlantic salmon (*Salmo salar*)

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Abstract

Legumes such as soybean, peas and lupin have attracted considerable interest as potential sources of protein to replace finite and limiting supplies of marine fishmeal (FM) as major ingredients for aquafeeds. In this respect, faba beans (*Vicia faba*) represent a widespread and relatively unexploited legume crop in Europe with potentially favourable characteristics. However, for carnivorous species such as Atlantic salmon (*Salmo salar*), protein levels in legumes are generally too low and require to be concentrated to be direct replacements for FM. Previously we showed that a faba bean protein concentrate, produced by air classification, containing 55 % protein could partially replace FM and/or soy protein concentrate (SPC) in feeds for parr and post-smolt salmon. In the present study, a faba bean protein isolate (BPI), produced by a wet process, with almost ~80 % crude protein was investigated in feeds for Atlantic salmon in seawater. Four dietary treatments were tested including one with high inclusion of FM (400 g kg⁻¹) and three with low FM (216 g kg⁻¹) and increasing inclusions of BPI (0, 70 and 140 g kg⁻¹) substituting for SPC (236, 125 and 45 g kg⁻¹). Growth performance in fish was unaffected with the lower level of dietary BPI, but was reduced in fish fed the higher level, mainly due to feed intake being reduced initially. Histological analysis of the distal intestine showed inflammation in fish fed both diets containing BPI, but especially at 140 g kg⁻¹. The high dietary level of BPI affected the transcriptome of pyloric caeca with almost 2000 differentially expressed genes (DEG) compared to fish fed FM, whereas fish fed SPC or the lower level of BPI showed no DEG compared to fish fed FM. In contrast, the liver transcriptome was generally affected similarly by both BPI and SPC. The combined data suggested that the BPI utilised contained a factor that was detrimental above a certain threshold and, although this factor could be an artefact of the protein isolation method, an effect of a known ANF could not be excluded with saponin the most likely candidate. Overall, however, the results of the present study confirmed that protein concentrates or isolates derived from faba beans can replace FM and/or SPC up to a certain level in feeds for Atlantic salmon.

1. Introduction

Carnivorous fish species such as Atlantic salmon (*Salmo salar*) have a high dietary requirement for protein (National Research Council, 2011) and therefore their culture is highly dependent on reliable supplies of dietary protein sources with a high nutritional value; that is, with good digestibility and a balanced amino acid composition. Fishmeal (FM), the most balanced and traditionally utilised source of protein for fish, is a finite resource and so the limited supply and high demand has resulted in increased prices. In modern fish feed, FM is generally used sparingly and strategically in favour of more available plant materials (Ytrestøyl et al., 2015). The suitability of plant proteins has been generally limited by the lower protein contents of plant meals and concentrates (20-60 %) relative to FM (~ 70 %), amino acid imbalances including lower availability of methionine and lysine among others, and presence of anti-nutritional factors (ANFs) (Gatlin et al., 2007; Krogdahl et al., 2010). However, the use of crystalline amino acids in the formulation of fish feeds to balance nutritional deficiencies (Nunes et al., 2014) and continual improvement of processing technologies for the production of more refined protein concentrates have made available an increasing number of ingredients that can be used as protein sources for salmon feed.

Among plant products, legumes such as soybean, lupin and peas have attracted considerable interest (Gatlin et al., 2007) and soybean protein concentrate (SPC) is currently one of the main protein sources used for the formulation of feeds for Atlantic salmon (Ytrestøyl et al., 2015). However, due to price volatility of raw materials such as FM and SPC novel protein concentrates with favourable characteristics are sought after to allow higher flexibility in least cost formulation. In this context, higher demand for locally produced legumes is expected. A widespread and relatively unexploited legume crop in Europe is faba bean (*Vicia faba*) (FAO STAT, 2009). Favourable characteristics of faba bean include low levels of ANF, with most concentrated in the seed coat that can be removed in the process of de-hulling (Vidal-Valverde et al., 1998; El-Shemy et al., 2000). Additionally, faba bean can be successfully used in crop rotation to help reduce the use of nitrogen fertilisers derived from fossil fuel sources.

Faba beans have a substantially lower protein content (~25 %) compared with soybean (~35 %) (El-Shemy et al., 2000), but whole crushed beans are currently utilised in commercial feeds for salmon in minimal quantities (~5 %), essentially as a starch source.

However, further processing of faba beans has provided ingredients with higher protein concentration as potential protein sources for salmonids. For example, de-hulling of faba bean before crushing produced a meal with $\sim 28\%$ protein that was tested as a replacement for soybean meal ($\sim 44\%$ protein) in rainbow trout fingerlings (Ouraji et al., 2013). This study demonstrated that rainbow trout fingerlings could tolerate up to 30% inclusion of faba bean meal in the diet and that inclusion levels of 15 % were beneficial for growth performance. A more attractive ingredient for feed formulation, with substantially higher protein content ($\sim 61\%$ crude protein), was produced by air-classification of de-hulled, crushed faba beans and investigated in feeds for Atlantic salmon in a screening trial using parr (De Santis et al., 2015a,b; Krol et al., 2016). The study on parr showed that salmon could efficiently utilise air-classified faba bean protein concentrate (BPC) up to inclusion levels of 26 % of feed (40 % of the protein fraction) without loss of performance when compared to feeds using the traditional protein ingredients, FM and SPC (De Santis et al., 2015a). Indeed, similar to the results reported in rainbow trout fingerlings, moderate inclusions of BPC appeared to be beneficial for growth of salmon parr. The study in salmon parr also indicated that high inclusions of BPC was marginally detrimental for the digestive tract causing a mild inflammation of the posterior intestine and also resulted in a significant loss of growth performance. A subsequent study using larger (~ 1.5 kg) salmon in seawater confirmed that a moderate inclusion level of around 21 % of feed of BPC (containing approximately 55 % crude protein) supported good growth in Atlantic salmon (De Santis et al., 2016).

In the present study, a wet processed faba bean isolate (BPI) with higher protein content ($\sim 80\%$ crude protein) and lower levels of ANF than BPC was investigated in feeds for Atlantic salmon. The BPI was produced using wet processing methodology involving aqueous alkaline solubilisation and acid precipitation adapted from methods used for the production of legume, including pea and lupin, protein isolates (Gueguen, 1983; Swanson et al., 1990; Jayasena et al., 2011). In the present study, we investigated the effects of BPI on growth and performance of Atlantic salmon in a trial using practical feed formulations and growing conditions in seawater to simulate commercial production. Four dietary treatments were tested including one treatment with high inclusion of FM (400 g kg^{-1}) and three treatments with identical low FM content (216 g kg^{-1}) and increasing inclusions of BPI (0, 70 and 140 g kg^{-1}) substituting SPC (236 , 125 and 45 g kg^{-1}). To evaluate performance, weight, length, feed intake, biochemical composition of the fillet, and distal

intestine histology were evaluated. In addition, transcriptomic analyses were performed in liver and pyloric intestine to determine the tissue-specific responses in gene expression to the dietary ingredients.

2. Material and Methods

2.1 Diet formulations and compositions of ingredients and feeds

The proximate and amino acid compositions of the three protein ingredients, FM (NA LT-70), SPC (60%) and BPI (KMC, Brande, Denmark), used to formulate the feeds are shown in Table 1. The combined vicine plus convicine content of BPI at 0.13 % (wt/wt of BPI) was almost 8-fold lower compared to the vicine/convicine content of the previously tested BPC (1.02 % wt/wt of BPC). Four dietary treatments included a control diet (named FM), formulated with the highest level of FM (400 g kg⁻¹), and three experimental diets, formulated with the same content of FM (216.4 g kg⁻¹) with one diet containing SPC (236 g kg⁻¹) and no BPI (named SPC) and two diets with BPI inclusions of 70 and 140 g kg⁻¹ (named diets BPI7 and BPI14) substituting primarily SPC (125 and 45 g kg⁻¹). The extruded feeds were isonitrogenous (45 % crude protein), isolipidic (20 % crude fat) and isoenergetic (23 MJ kg⁻¹), and formulated to meet the nutritional requirement of salmon (National Research Council, 2011). The formulations were designed to generally reflect current commercial formulations in terms of type and inclusion levels of other ingredients (Table 2).

2.2 Fish feeding trial and sampling

The nutritional trial was conducted at Marine Harvest (Scotland) Ltd. Feed Trial Unit (Ardnish, Lochailort, UK) using autumn smolt (0+) Atlantic salmon (*Salmo salar*) of the commercial Aquagen strain (Aquagen Ltd, Kyrksæterøra, Norway), produced at the Marine Harvest freshwater production unit (Glenfinnan, Argyll, Scotland). A total of 3000 fish were randomly allocated in batches of 250 fish to one of twelve 5m³ pens. The pens were supplied with automatic feeders that delivered two daily feeds (8 am to 10 am and 2 pm to 4 pm) to apparent satiation by oversupplying the feed by approximately 10 %. Feed delivered was recorded daily and uneaten feed collected by an uplift system throughout the experiment 30 min post feeding. For calculation of feed intake, uneaten feed was corrected for water content and subtracted from the feed delivered. Fish were

acclimatised for 4-weeks prior to application of the experimental feeds during which time they were all fed the same commercial feed (BioMar UK Ltd., Garngemouth, Scotland). At the start of the trial (November 2014), fish were bulk weighed (average weight 204 g) and returned to the pens. Each experimental feed was fed to triplicate pens for 11 weeks after which individual weights were measured for all fish, length for a subset of 50 fish *per pen*, and visceral/liver weights for a subset of 18 fish *per pen*. Samples of flesh (Norwegian Quality Cut, NQC) were collected from 5 fish *per pen* and immediately frozen on dry ice before being stored at -20 °C prior to biochemical analyses. For histological examination, distal intestine of 13 fish per replicated pen were fixed in phosphate buffered saline. In addition, samples of liver and pyloric caeca were dissected from 24 individuals *per* dietary treatment (8 per tank replicate) keeping the area of dissection consistent for every fish. Pyloric caeca were carefully cleaned of visceral fat, and luminal content was gently squeezed out. The tissue samples were immediately placed in RNA Later (Life Technologies, Paisley, UK) and processed as per manufacturer's instructions before being stored at -20 °C prior to molecular analyses.

2.3 Biochemical analyses

Proximate compositions of feeds and salmon flesh were determined according to standard procedures (AOAC, 2000). Flesh samples were skinned and boned and pooled *per pen* into 3 samples of 5 fish (1 pool *per pen* replicate) and homogenised in a blender (Waring Laboratory Science, Winsted, CT, USA) to produce pates, and feeds were ground prior to analyses. Moisture contents were obtained after drying in an oven at 110 °C for 24 h and ash content determined after incineration at 600 °C for 16 h. Crude protein content was measured by determining nitrogen content ($N \times 6.25$) using automated Kjeldahl analysis (Tecator Kjeltac Auto 1030 analyser, Foss, Warrington, U.K.), and crude lipid content determined after acid hydrolysis followed by Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus, Foss, Warrington, U.K.).

2.4 Vicine-convicine analysis

Vicine and convicine content of the BPI used in the present study and the BPC (Netsch GmbH, Selb Bavaria, Germany) used previously (De Santis et al., 2015a, b) were assessed. The analysis was carried out using an HPLC-based method (NIAB-TAG Ltd, Cambridge, UK), according to Khamassi et al. (2013), a modification of Lattanzio et al.,

1982). For each BPC sample, 0.5 g samples were extracted in sterile distilled water by vortexing and treating in an ultrasonic water bath at 40 °C for 30 min. After filtering (Whatman No. 1), the filtrate was diluted to 100 mL with sterile distilled water and an aliquot filtered using a 0.45 µm syringe disc-filter and separated on an HPLC system (Dionex Ultimate 3000 HPLC), equipped with a Phenomenex Spherclone ODS II column (250 x 4.6 mm x 5 µm) with sterile distilled water as the mobile phase at a flow rate of 1.5 mL min⁻¹ and eluent monitored with a diode array detector recording at 229 nm, 254 nm, 280 nm and 400 nm. Peaks were identified as vicine and convicine by their retention time relative to L-DOPA (L-dihydroxyphenylalanine) reference solutions.

2.5 Histological analysis

Tissue processing has been described previously by De Santis et al. (2015). Briefly, tissues were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin. A microtome was used to slice 5 µm transverse sections of the distal intestine samples. The sections were mounted onto microscope slides (4 sections per slide) and stained with haematoxylin, eosin (H&E) and Alcian Blue staining. The slides were digitalised with a Zeiss Axioscan Z1 slide scanner at x20 magnification, randomised images were scored blindly according to the semi-quantitative scoring system developed by Urán et al. (2009) on a scale from 1 (no enteritis) to 5 (severe enteritis) to assess the degree of SBM-induced enteritis through six parameters: sub-epithelial mucosa (SM), lamina propria (LP), eosinophilic granulocytes (EG), mucosal folds (MF), goblet cells (GC) and supranuclear vacuoles (SV).

2.6 Transcriptome analysis

Transcriptomic analysis was conducted using a custom-made 4 x 44K Atlantic salmon oligo microarray (Agilent Technologies, Wokingham, UK; ArrayExpress accession no. A-MEXP-2065) described in detail previously (Tacchi et al. 2011). Briefly, RNA was extracted from 50 mg of liver tissue or pyloric caeca using TRI Reagent (Sigma-Aldrich, Dorset, UK). Equal amounts of RNA from four fish from the same pen were extracted individually, pooled together and analysed as a single biological replicate, thus providing 6 replicates *per* dietary treatment. The resulting RNA samples were amplified using TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit, (Epicentre Technologies Corporation, Madison, Wisconsin, USA) following recommended procedures. Aminoallyl-amplified RNA (aRNA) samples were labelled with Cy3 dye (GE HealthCare

Life Sciences, Buckinghamshire, UK) while a pool of all aRNA samples was labelled with Cy5 dye (GE HealthCare Life Sciences) and was used as a common reference in a dual-label common reference design and hybridised to each array. Scanning was performed using a GenePix 4200 AL Scanner (Molecular Devices (UK) Ltd., Wokingham, UK), and the resulting images analysed with Agilent Feature Extraction Software v.9.5 (Agilent Technologies) to extract the intensity values and identify the features. Features considered outliers (*i.e.*, defined as those probes whose background intensity was between the 0.05th and 99.95th percentile of the distribution) in two or more replicates within at least one treatment were excluded from further analyses. Additionally, features consistently expressed just above background noise (defined as those features whose intensity was lower than 5th percentile of the distribution in 75% or more of the analysed samples) were also removed. The full protocol for microarray laboratory and data analysis has been previously reported (De Santis et al., 2015b). The output of the microarray experiment was submitted to ArrayExpress under accession number E-MTAB-4661.

2.6 Statistical and data analysis

All statistical and data analyses were performed using the software R v.3.2.1 (R Core Team, 2013). Feed intake, somatic and biochemical data were analysed using the function *lmer* from the package lme4 (Bates et al., 2015). Specifically, feed intake was analysed using a repeated measure linear effect model, where pens were accounted as random factor nested within treatments (*i.e.* feed) and days used as the repeated measure factor. The remaining data was analysed using linear mixed models including: *a*) final individual weight ($n = 750$, initial average fish size and treatments as fixed factors, pens nested within treatment as a random factor); *b*) individual length ($n = 150$, treatments as fixed factor, pens nested within treatments as random factor); *c*) visceral somatic index ($VSI = \text{visceral weight} / \text{body weight} * 100$; $n = 39$, treatments as fixed factor, pens nested within treatments as random factor) and hepatic somatic index ($HSI = \text{liver weight} / \text{body weight} * 100$; $n = 39$, treatments as fixed factor, pens nested within treatments as random factor). Feed conversion ratio (FCR) was calculated on bulk data from the whole pen using the formula $FCR = \text{total feed consumed} / (\text{final weight} - \text{initial weight} + \text{weight lost through mortalities})$. Detailed results of the statistical analysis are provided as Supplementary Table 1.

For histology of the distal intestine, score spread for each parameter was visualised in R through boxplots and analysed with the Rao-Scott Cochran-Armitage by Slices (RSCABS) method implemented in the R package StatCharrms (Green et al., 2014). This method analyses each parameter separately and slices through the data by severity level. The counts of fish with a given severity level are compared to the count of fish for that level found in the control FM diet. If the count was found to be significantly higher in the respective test plant diet, there is indication that the diet induced enteritis for the given parameter at the identified severity level.

Transcriptomic data analysis was performed using Bioconductor v.2.13 (Gentleman et al., 2004). Quality control, data pre-processing and analysis of differential expression were conducted using the software package limma (Smyth, 2004). To avoid redundancy, features representing the same target gene as implied from KEGG annotation were reduced into a unique value obtained by selecting the feature with the highest F-value calculated on all contrasts. For analysis of gene expression we adopted gene-set testing using the function *roast* of the limma package (Wu et al., 2010). Gene set testing is a differential expression analysis in which a set of *a priori* defined (putatively co-regulated) genes is treated as a unit. Gene set testing allows focusing attention on biologically meaningful processes and provides a more powerful and robust approach than traditional gene-wise tests as evidence is accumulated from many genes. All *p*-values reported in this work were corrected for false discovery rate (Benjamini and Hochberg, 1995).

3. Results

3.1 Feed intake and FCR

Feed intake in fish fed the FM, SPC and BPI7 diets was initially high in the first two weeks of the experiment but then gradually decreased as water temperature declined (Fig. 1). Fish fed BPI14 showed slightly lower feed intake at 1 week and thereafter the reduction in intake as temperature decreased was more rapid than in fish fed the other feeds. By week 5 there were no significant differences in feed intake as % of body weight amongst experimental treatments. Over the experimental period, fish fed BPI14 consumed significantly less feed than fish fed the other treatments, and fish fed BPI7

showed higher intake compared with those fed the control diet FM. There were no statistical differences in FCR between fish fed FM, SPC and BPI7 whereas FCR of fish fed treatment BPI14 was significantly higher (Fig.1).

3.2 Somatic data and biochemical composition

At the end of the 11-week experimental period fish had grown from an average of 203 g to 570 g. Statistical analyses (Supplementary Table 1) indicated that the BPI14 feed negatively affected performance with a loss of weight gain of up to 166 g and 1.4 cm compared with fish fed SPC that showed the highest average weight (Fig. 2). No statistically significant differences in final weight were detected between fish fed SPC and those fed BPI7, which both had marginally higher final weights (~30 g and 25 g higher for fish fed SPC and BPI7, respectively) than fish fed diet FM. Fish fed BPI14 also had significantly higher visceral and hepatic weights relative to body weight compared to fish fed the other treatments. Although HSI was not statistically different between fish fed BPI14 and BPI7, both were higher than fish fed FM (Table 3). Protein and ash contents of the flesh were not affected by dietary treatment but lipid (oil) content was lower (and moisture higher) in fish fed BPI14 compared to fish fed the other diets (Table 3).

3.3 Distal intestine histology

Distal intestine of fish fed the experimental diets all showed varying levels of inflammation compared to the fish fed the FM diet (Fig. 3). Fish fed the SPC diet showed a mild increase in LP, EG and SV compared to fish fed FM, although these were significantly increased it was generally less than one score criterion. In contrast, dietary BPI appeared to induce more severe levels of enteritis in a significantly higher number of fish and for a wider range of assessed parameters. Distal intestine of fish fed diet BPI14 had higher scores than fish fed BPI7 (Fig. 4). The most severe effects were found for SV, where both BPI diets showed significantly higher numbers of fish scoring in severity levels 2 to 5 compared to fish fed FM, with higher significance levels in the BPI14 group (Table 4). A similar situation was found for the LP and EG parameter for levels 3 and 4. Regarding the MF parameter, fish fed BPI7 showed increased score compared to fish fed FM and, with fish fed BPI14, a significantly higher number of fish reached severity level 5. Dietary effect on sub-mucosal gaps was minimal and no effect of diet on the distribution of goblet cell severity scores was identified.

3.4 Liver transcriptome

The liver transcriptome was significantly affected in fish fed all experimental feeds with 903 (SPC), 189 (BPI7) and 798 (BPI14) differentially expressed genes ($p < 0.05$) when compared with the FM control. This translated into 34 (SPC), 16 (BPI7) and 64 (BPI14) gene-sets that were differentially expressed ($q < 0.05$) compared with fish fed FM (see Supplementary Table 2). Therefore, although the number of hepatic genes differentially regulated in fish fed BPI14 compared to SPC was lower, they spanned a larger number of gene-sets (Fig. 5). These comprised especially metabolic pathways, which were significantly more affected in fish fed BPI14 compared to fish fed both SPC and BPI7. Indeed, in fish fed BPI7 only a few gene-sets/pathways were affected (Fig. 5, Supplementary Table 2). The data revealed generally reduced expression of metabolic pathways in fish fed BPI14 and, to a lesser extent, SPC, compared to fish fed FM. Where affected, the direction of change of gene-sets was consistent between treatments. Processes that were similarly affected in all treatments included increased immune system, signalling molecule and interactions, and signal transduction gene-sets, and reduced carbohydrate metabolism and glycan metabolism, especially glycosyl phosphatidyl inositol (GPI)-anchor biosynthesis. Gene-set data was confirmed by individual gene expression data, which is reported as Supplementary Fig. 1.

3.5 Pyloric caeca transcriptome

Feed SPC and BPI7 did not alter the transcriptome of the pyloric intestine compared with FM. In contrast, when fish were fed BPI14 a significant alteration of the caecal transcriptome was observed which was explained by 1989 genes differentially expressed (0 and 1 gene affected in SPC and BPI7 respectively). At gene-set level this translated into 93 differentially expressed gene-sets/pathways ($q < 0.05$) in BPI14 when compared with the FM control (see Supplementary Table 3). The response in the pyloric intestine observed after feeding treatment BPI14 involved a clear directional pattern of the main biological processes (Fig. 6). Specifically, gene-sets of the organismal system (*i.e.* immune, excretory, digestive, endocrine systems) and environmental information processing (*i.e.* signalling molecules and interaction, signal transduction, membrane transport) were expressed at lower levels in fish fed BPI14 compared with fish fed FM. On the contrary, gene-sets of metabolism and genetic information processing were higher expressed in fish fed BPI14 compared with fish fed FM. Among cellular processes, cell

motility transport and catabolism, and cell communication were lower expressed whereas cell growth and death was higher expressed in BPI14 compared with FM (Fig. 6). Gene-set data was confirmed by individual gene expression data, which is reported as Supplementary Fig. 2.

4. Discussion

The present study investigated growth performance, biochemical composition and tissue gene expression in response to dietary BPI. Three diets were investigated that had identical FM levels (216 g kg^{-1}) and increasing inclusions of BPI (0, 70 and 140 g kg^{-1} , respectively) substituting SPC (236 , 125 and 45 g kg^{-1} , respectively). A further treatment with higher inclusions of FM (400 g kg^{-1}) was also included as a control or reference feed. The results indicated that moderate amount of BPI (70 g kg^{-1} , contributing 16 % of total protein) could substitute for SPC without any significant loss of growth performance, alteration of biochemical parameters or major metabolic responses in liver or pyloric intestine. However, increasing the dietary inclusion level of BPI to 140 g kg^{-1} (32 % of total protein) resulted in reduced growth performance, increased FCR, altered flesh fat content, a marked transcriptomic response in pyloric intestine and increased levels of intestinal inflammation.

The reduced growth performance could be at least partially attributed to reduced feed intake, especially evident during the initial five weeks of the feeding trial. It is possible that the BPI utilised contained a factor that affected palatability or provoked an adverse reaction once consumed. One candidate could be a residual artifact of the processing methodology. The wet process for producing protein isolates from legumes generally involves protein extraction with alkaline solution followed by acid precipitation (Gueguen, 1983; Swanson, 1990). At the latter stage the pH of the solubilized protein solution is reduced with sulphuric acid and the precipitated protein recovered by centrifugal decanter before being dried. Total sulphate in the BPI was high at $\sim 5 \text{ g kg}^{-1}$ (data not shown) but would only contribute about 0.7 g kg^{-1} to the feed in the high BPI formulation, with “high sulphate” foods classified as containing $>1 \text{ g kg}^{-1}$ (Florin et al., 1993). However, dietary sulphate is ubiquitous in foods and not regarded as harmful or toxic, and there is no evidence that sulphate in feed would provoke an adverse reaction in

salmon. Residual acidity could perhaps affect palatability but is unlikely to have a negative impact thereafter in a monogastric animal with an acidic stomach.

The most obvious components in plant protein products likely to cause issues with feed intake are ANFs (Gatlin et al., 1997). In faba bean, quantitatively the main ANFs are the soluble sugars, α -galactosides, such as raffinose, stachyose and verbascose (Sosulski and Cadden, 1982). There are few data on the effects of these oligosaccharides in fish but one study in Atlantic salmon investigating the effects of raffinose, stachyose, or a combination of the two, showed they provoked no morphological changes in liver, or mid and distal intestines and did not interfere with protein or fat digestibility (Sørensen et al., 2011). In addition, the soluble nature of the oligosaccharides suggests that they will most likely be lower in BPI than in BPC as soaking decreased α -galactoside levels in faba beans (Vidal-Valverde et al., 1998). Major ANFs more specific to faba beans are the pyrimidine glycosides, vicine and convicine, that accumulate in the cotyledons (Lattanzio et al., 1983; Khamassi et al., 2013). Pyrimidine glycosides have been shown to have various deleterious effects in chickens, including decreased food consumption and weight gain (Marquardt et al., 1976; Muduuli et al., 1982), and the anti-nutritional effects of vicine and convicine in faba beans used in feeds for pigs, poultry and ruminants have been reviewed (Crepon et al., 2010). In contrast, no negative effects of dietary vicine/convicine in fish have been reported. Rainbow trout did not show any significant reduction of feed intake and could tolerate dietary inclusion of FBM up to 450 g kg⁻¹ (Ouraji et al., 2013). Similarly, Atlantic salmon in both freshwater and seawater did not present evidence of detrimental effects or reduced palatability when fed inclusions of BPC comparable with those of BPI used in the present study (De Santis et al., 2015a, 2016). Furthermore, the BPI used in the present study had levels of vicine/convicine combined that were around 8-fold lower than those measured in the previously used BPC. Therefore, the wet process used for the production of BPI largely removed these ANF, consistent with reports that vicine/convicine could be extracted by steeping beans in an acid bath (Marquardt et al., 1983). Therefore, it is unlikely that negative effects such as decreased feed intake and intestinal inflammation were due to the very low levels of vicine and convicine. Other relevant ANFs are saponins that are known to be, at least partly, responsible for the negative effects of dietary soybean meal in salmonids, including reduced growth performance and severe inflammation in the distal intestine (Knudsen et al., 2008; Sørensen et al., 2011; Krogdahl et al., 2015). The saponin contents

of faba beans and meal, at around 4.3 g.kg^{-1} , are an order of magnitude lower than levels in soybean (43 g.kg^{-1}) (Fenwick and Oakenfull, 1983). However, whereas saponins were undetectable in SPC produced by ethanol extraction, they were higher in a soy protein isolate produced by isoelectric precipitation (Ireland et al., 1986). Similarly, a faba bean protein isolate showed higher saponin content than the meal although the method of preparation of the isolate was not given (Fenwick and Oakenfull, 1983). Although saponin content was not measured in the present study it is possible that it may have been higher in the BPI compared to the earlier BPC and so it may have contributed to the negative effects observed in fish fed BPI14.

The responses of key metabolic tissues provide another strategy to gain some insight to the effects of diet. In the present study we investigated transcriptomic responses in pyloric intestine, as a tissue directly in contact with feed and so potentially responding to ingredients/raw materials, and liver as the tissue responding to dietary nutrients. Certainly, the transcriptomic responses in the two tissues were different. In liver, SPC and BPI14 showed the greatest response compared to fish fed FM, with BPI7 provoking a much lower response. This was consistent with previous data that showed when FM was replaced by a single plant protein ingredient (as in SPC and BPI14 feeds in the present study), transcriptomic responses in liver (De Santis et al., 2015b) and distal intestine (Krol et al., 2016) were greater than when combinations of plant proteins were used, as in BPI7 in the present study. Generally, metabolic pathways were the most affected showing reduced expression in liver of fish fed all experimental feeds compared to fish fed FM. Other pathways were also generally similarly affected by all experimental feeds with genes of immune system, signalling molecule and interactions, and signal transduction showing increased expression, and carbohydrate metabolism and glycan metabolism, showing reduced expression. This showed some consistency with hepatic processes significantly affected in salmon parr fed high levels of BPC that included digestive functions (carbohydrate digestion and absorption, and pancreatic secretion), the immune response (complement and coagulation cascades), and amino acid metabolism (tyrosine and beta-alanine) (De Santis et al., 2015b).

In contrast, it was evident from the analysis of the pyloric caeca transcriptome that a relatively large response was observed after feeding BPI14 whereas there was no major response to either SPC or BPI7 when compared to the FM diet. Thus, fish fed BPI14 showed over 90 differentially expressed gene-sets compared with fish fed FM and, while

genes of metabolic, cell growth and death processes showed increased expression, most gene sets including immune, excretory, digestive, endocrine systems, signalling molecules and interaction, signal transduction and membrane transport were expressed at lower levels. Pyloric caeca are an important site for nutrient absorption (Grosell et al., 2011) and it has long been known that ANF such as phytic acid can affect the morphology of the gastrointestinal tract of fish resulting in hypertrophy and increased vacuolation of pyloric caeca (Hossain and Jauncey, 1993). Faba bean contain phytic acid (Vidal-Valverde et al., 1998), and this could have been increased in BPI as it follows the protein, as has been shown in soybean where the meal contains ~4 % but levels can be as high as 7-10 % in SPC (Gatlin III et al., 2009). However, as the response observed in pyloric caeca of fish fed BPI was much greater than in fish fed SPC, it was unlikely to be due to phytic acid. However, the transcriptomic response in pyloric caeca gave no obvious clues the chemical identity of the factor provoking the adverse reaction, which was perhaps not surprising. There are few studies investigating the molecular response of pyloric intestine to reinforce the present data (Morais et al., 2012; Betancor et al., 2015; De Santis et al., 2015c) as most studies on the effects of dietary plant proteins have focussed on distal intestine (Tacchi et al., 2011, 2012; Kortner et al., 2012; Sahlmann et al., 2013; De Santis et al., 2015d; Krol et al., 2016). In salmon fed increasing levels of soybean meal that provoked increasing enteritis, digestive enzyme activities in distal intestine were decreased, as observed in the present study (Krogdahl et al., 2003). However, in the earlier study the response in mid intestine was different with some enzymes showing a similar pattern to that in distal intestine, but others showing a less consistent response.

Although fish fed both the BPI7 and BPI14 diets showed increased histological scores, the pattern of inflammation observed was different from the inflammation found in distal intestine of salmon fed the high levels of BPC previously, where only GC and SV were altered. In this case GC was not significantly changed whereas most of the other parameters were increased. It should be noted that the fish in the present study were much larger than those described by De Santis et al. 2015a and were also maintained in seawater compared to freshwater, however it may be that different factors caused the inflammation in response to dietary BPI than the high levels of BPC. This could be consistent with the above discussion regarding saponin contents that may be higher in BPI than in BPC. The saponin-related enteritis observed with soybean meal is

characterized by shortening of the mucosal folds, infiltration of the lamina propria by inflammatory cells, and decreased numbers of absorptive vacuoles in enterocytes (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003, 2015). Interestingly, in the present study, the most striking change in the distal intestine was deterioration of the absorptive vacuoles with increasing BPI, almost absent in fish fed BPI14. Lamina propria were also significantly widened in fish fed BPI, possibly which linked to increased infiltration of eosinophilic granulocytes, and shortening of the folds was also significant and appeared dose dependent with BPI. Therefore, the changes observed and symptoms of progressing enteritis were similar, at least on a histological level, to those observed in soybean (saponin)-induced enteritis.

The results of the present study have confirmed that protein concentrates or isolates derived from faba beans can replace FM and SPC up to a certain level in diets for Atlantic salmon. In previous studies using BPC, this level was about 200 g kg⁻¹ of diet representing about 110 g or 28 % of dietary protein. With the BPI, a similar level of inclusion affected feed intake in the first 5 weeks and this impacted growth although feed intake later stabilised. The lower inclusion level of BPI gave no detrimental effects on growth performance as had previously been obtained with BPC, but there was evidence of mild / moderate intestinal inflammation and the higher level induced more severe enteritis. Thus, the data suggested that the BPI utilised contained a factor that was detrimental above a certain threshold, becoming important when dietary inclusion reached 140 g kg⁻¹. This may be an artefact of the protein isolation method, but could be associated with an ANF and, in this respect, saponin was the most likely candidate. However, the overall conclusion was that, depending upon processing method, specific protein concentrates or isolates from faba bean can replace a proportion of FM and/or SPC in feeds for Atlantic salmon.

Acknowledgements

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Legends to Figures

Figure 1. Feed intake calculated as weekly average of feed consumed as percent of body weight per day (*y*-axis). Weekly average of daily temperature measurements are reported on *x*-axis. The grey box represents the corresponding feed conversion ratio (FCR) for the treatments over the 11 week experimental period. Feed intake was significantly lower in fish fed diet BPI14 than in fish fed the other feeds in the first 5 weeks and, other than this there were no significant differences in feed intake. For FCR, same letters denote no statistical differences. In both graphs, bars denote standard error of the mean (*n* = 3) for the replicate pens.

Figure 2. Summary of somatic data. a) Weight distribution; b) Length distribution.

Figure 3. Cross-sections of distal intestine from Atlantic salmon fed the four experimental feeds. (A) FM diet: no inflammation, healthy SV and GC, long MFs with slender LP and no infiltration of EG. (B) SPC diet: low enteritis scores, healthy SV and GC, slightly shorter MFs with slightly enlarged LP due to increased migration of EG. (C) BPI7 diet: medium enteritis scores, reduced SV, shortening of MF and increased SM, LP widened with a high number of migrating EG. (D) BPI14 diet: high enteritis cores, disappearance of SV, severe tissue damage with destruction of MFs, enlarged SM, greatly widened LP and large clusters of migrating EG. EG, eosinophilic granulocytes (significantly increased numbers in all plant diets, most severe in BPI diets); GC, goblet cells (no significant difference between diets); LP, lamina propria (significantly widened in all plant diets, most severe in BPI diets), MF, mucosal folds (significantly shortened/lost in BPI diets); SM, sub-mucosa (significantly increased area in both BPI diets); SV, supranuclear vacuoles (significantly reduced in all plant diets, most severe reduction and near complete loss in BPI diets).

Figure 4. Boxplots of factors scored for the assessment of the progress of intestinal enteritis in Atlantic salmon fed diets with different inclusion levels of plant protein. FM, fishmeal; SPC, soybean protein concentrate; BPI7, low bean protein isolate; BPI14, high bean protein isolate. A = Sub-mucosal gap, B = Mucosal folds, C = Lamina propria, D = Eosinophilic granulocytes, E = Goblet cells, F = Supranuclear vacuoles.

Figure 5. Summary of gene sets differentially expressed in response to dietary treatments in the liver. Only gene sets that were statistically different (up or down-regulated) in

dietary treatments compared with FM control are plotted. Bars represent standard deviation from the mean (absence of bar indicates that a single gene-set was present in the specific functional group). Gene sets are grouped by functional hierarchies as per KEGG classification (Kanehisa et al., 2012). Full details on individual gene sets affected in liver are provided as Supplementary Table 2. A selection of the most differentially expressed genes in the liver is provided as Supplementary Figure 1.

Figure 6. Summary of gene sets differentially expressed in response to dietary treatments in the pyloric caeca. Only gene sets that were statistically different (under or over expressed) in dietary treatments compared with FM control are plotted, however no gene sets were affected in response to SPC and BPI7 hence only BPI14 is plotted. Bars represent standard deviation from the mean (absence of bar indicates that a single gene-set was present in that specific functional group). Gene sets are grouped by functional hierarchies as per KEGG classification (Kanehisa et al., 2012). Full details on individual gene sets affected in liver are provided as Supplementary Table 3. A selection of the most differentially expressed genes in the liver is provided as Supplementary Fig. 2.

Table 1. Proximate and amino acid compositions

(expressed as %, unless otherwise specified) of the main protein ingredients utilised.

Nutrient	FM	SPC	BPI
Moisture	7.7	7.5	10.0
Protein (crude)	68.3	59.2	77.8
Lipid (crude)	9.5	2.8	4.9
Ash	15.5	6.2	2.6
Phosphorus (total)	2.16	0.70	0.55
Arginine	3.82	4.18	6.09
Histidine	2.07	1.49	1.75
Isoleucine	2.74	2.66	3.41
Leucine	4.89	3.56	6.30
Lysine	5.28	3.56	5.37
Methionine	1.84	0.78	0.83
Phenylalanine	2.73	2.98	4.13
Threonine	2.89	2.40	2.79
Tryptophane	0.81	0.77	0.40
Valine	3.33	2.84	3.61
Cysteine	0.56	0.74	0.78
Tyrosine	2.14	2.24	2.54

Table 2. Formulation (g kg⁻¹) and analysed proximate compositions of the experimental diets

	FM	SPC	BPI7	BPI14
<i>Ingredients</i>				
Fishmeal	400.0	216.4	216.4	216.4
Soy Protein Concentrate	19.5	236.3	125.1	44.7
Bean Protein Concentrate	0.0	0.0	70.0	140.0
Sunflower Expeller	75.8	5.3	42.0	57.9
Wheat Gluten	84.6	97.9	92.7	81.5
Maize Gluten	50.0	50.0	50.0	50.0
Wheat	57.5	56.0	59.9	65.7
Horse Beans	68.9	54.2	65.5	69.5
Fish Oil	94.1	107.7	105.5	102.9
Rapeseed Oil	127.4	132.1	129.2	127.0
Vitamin, Mineral and Pigment Premix	10.7	10.7	10.7	10.7
Amino Acid Mix	11.1	33.2	32.6	33.3
Yttrium	0.5	0.5	0.5	0.5
<i>Proximate Composition</i>				
Protein - crude (%)	45.4	44.3	43.6	45.0
Fat - crude (%)	22.3	20.5	20.3	23.9
Ash (%)	8.1	6.9	6.8	6.5
Energy - gross (MJ kg ⁻¹)	23.2	23.0	23.4	24.0

Table 3. Feed utilisation, growth and somatic indices of fish, and biochemical composition of flesh

	FM	SPC	BPI7	BPI14
FCR	0.88 ± 0.03 ^a	0.87 ± 0.02 ^a	0.91 ± 0.03 ^a	0.98 ± 0.05 ^b
TGC	3.97 ± 0.03 ^a	4.23 ± 0.12 ^a	4.16 ± 0.11 ^a	2.97 ± 0.03 ^b
HSI	1.46 ± 0.23 ^a	1.56 ± 0.17 ^{ab}	1.68 ± 0.28 ^{bc}	1.79 ± 0.32 ^c
VSI	12.46 ± 1.38 ^a	12.26 ± 1.57 ^a	12.80 ± 1.34 ^a	14.95 ± 1.96 ^b
Protein	19.85 ± 0.36	19.49 ± 0.39	19.42 ± 0.32	19.84 ± 0.55
Lipid	12.89 ± 1.33 ^a	12.88 ± 1.23 ^a	13.39 ± 0.71 ^a	9.14 ± 0.77 ^b
Moisture	66.25 ± 1.06 ^a	65.89 ± 1.00 ^a	65.69 ± 0.49 ^a	69.27 ± 1.51 ^b
Ash	1.88 ± 0.04	1.84 ± 0.06	1.86 ± 0.06	1.74 ± 0.21

Mean values and standard deviation (±SD) are presented for each parameter (n = 3). Means in the same row with different letters are significantly different ($p < 0.05$). FCR, food conversion ratio; HSI, hepato-somatic index; VSI, visceral-somatic index; TGC, thermal growth coefficient.

Table 4. Diagnostic features of intestinal enteritis sliced by severity score based on comparisons of A) the plant-based diets SPC (soybean protein concentrate), BPI7 (low bean protein isolate) and BPI14 (high bean protein isolate) to the FM (fishmeal) control diet and, B) bean-based diets BPI7 (low bean protein isolate, 7%) and BPI14 (high bean protein isolate) to the SPC (soybean protein concentrate) diet. P-values were calculated with the Rao-Scott Cochran-Armitage by Slices (RSCABS) procedure.

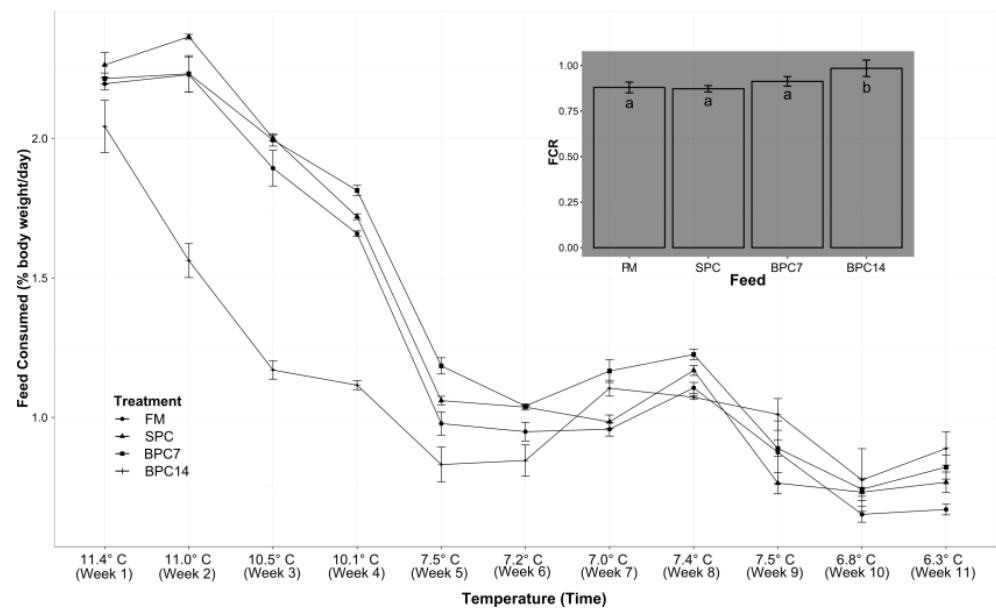
A)

Feature	Score	SPC (p-value)	BPI7 (p-value)	BPI14 (p-value)
Sub-mucosal gap	2	not significant	0.00023	0.00001
Mucosal folds	3	not significant	not significant	0.00156
	4	not significant	< 0.00001	< 0.00001
	5	not significant	not significant	< 0.00001
Lamina propria	3	0.02985	0.00039	0.00003
	4	not significant	< 0.00001	< 0.00001
Eosinophilic granulocytes	3	0.00913	0.00006	< 0.00001
	4	0.00004	< 0.00001	< 0.00001
Supranuclear vacuoles	2	0.02002	0.00634	0.00328
	3	0.00321	< 0.00001	< 0.00001
	4	not significant	< 0.00001	< 0.00001
	5	not significant	0.00256	< 0.00001

B)

Feature	Score	BPC7 (p-value)	BPI14 (p-value)
Sub-mucosal gap	2	0.00013	0.00015
Mucosal folds	3	0.02985	0.00039
	4	< 0.00001	< 0.00001
	5	0.03867	< 0.00001
Lamina propria	3	0.03867	0.01582
	4	< 0.00001	< 0.00001
Eosinophilic granulocytes	3	0.03867	0.01582
	4	0.03131	0.00009
Supranuclear vacuoles	3	0.00042	0.00001
	4	< 0.00001	< 0.00001
	5	0.01041	< 0.00001

19 Fig.1.



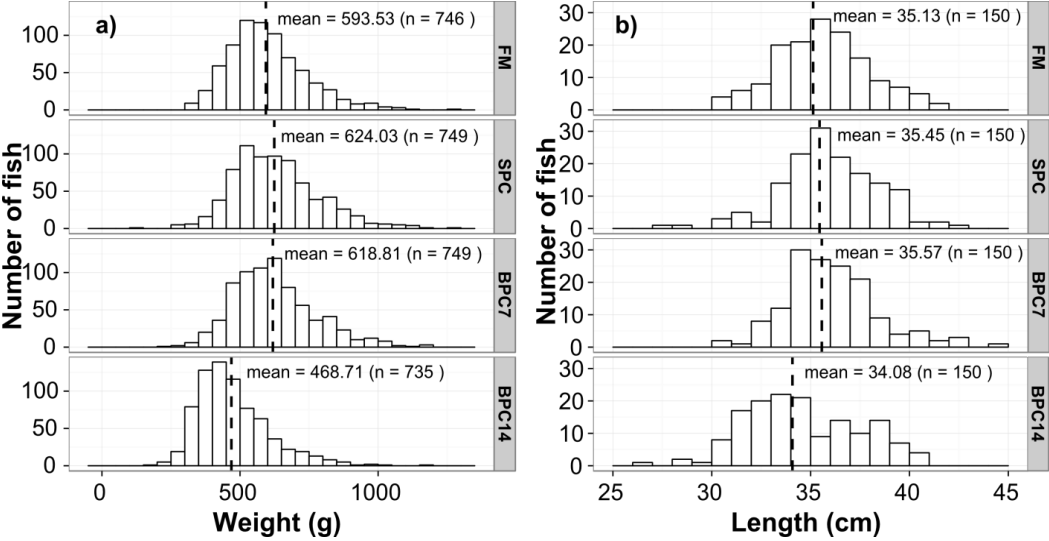
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23 Fig.2.

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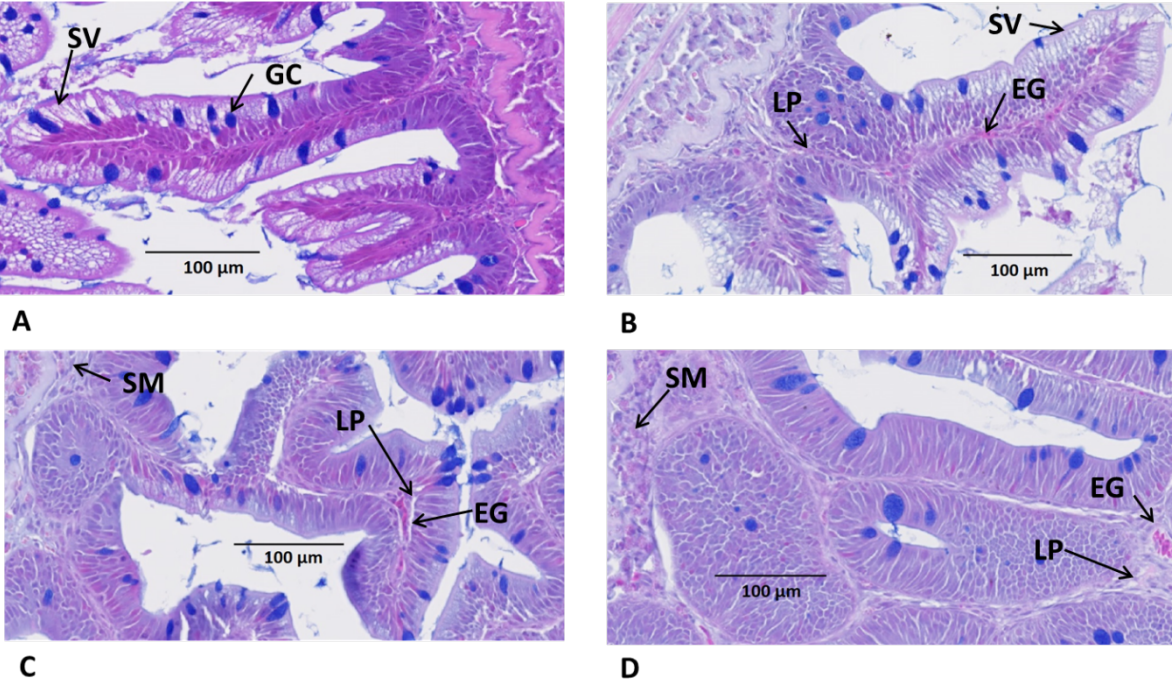
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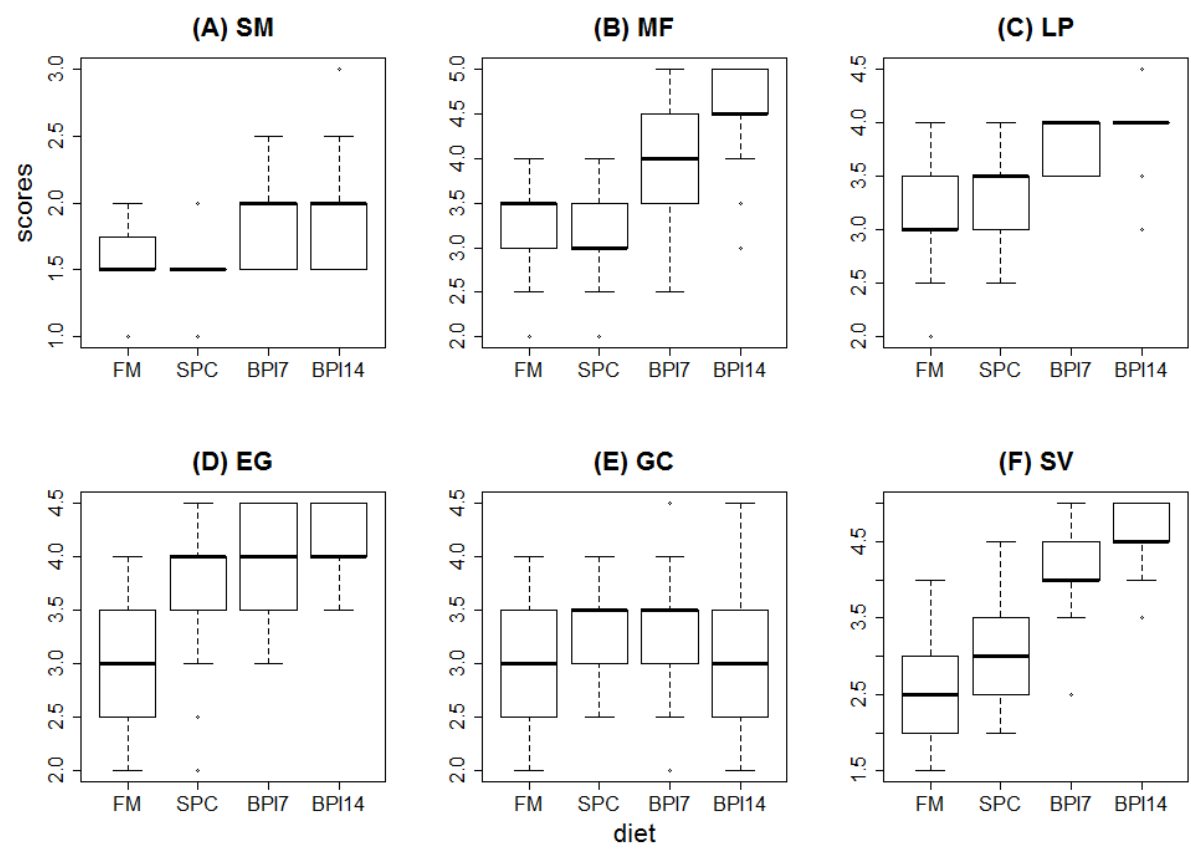
28

29 Fig.3.
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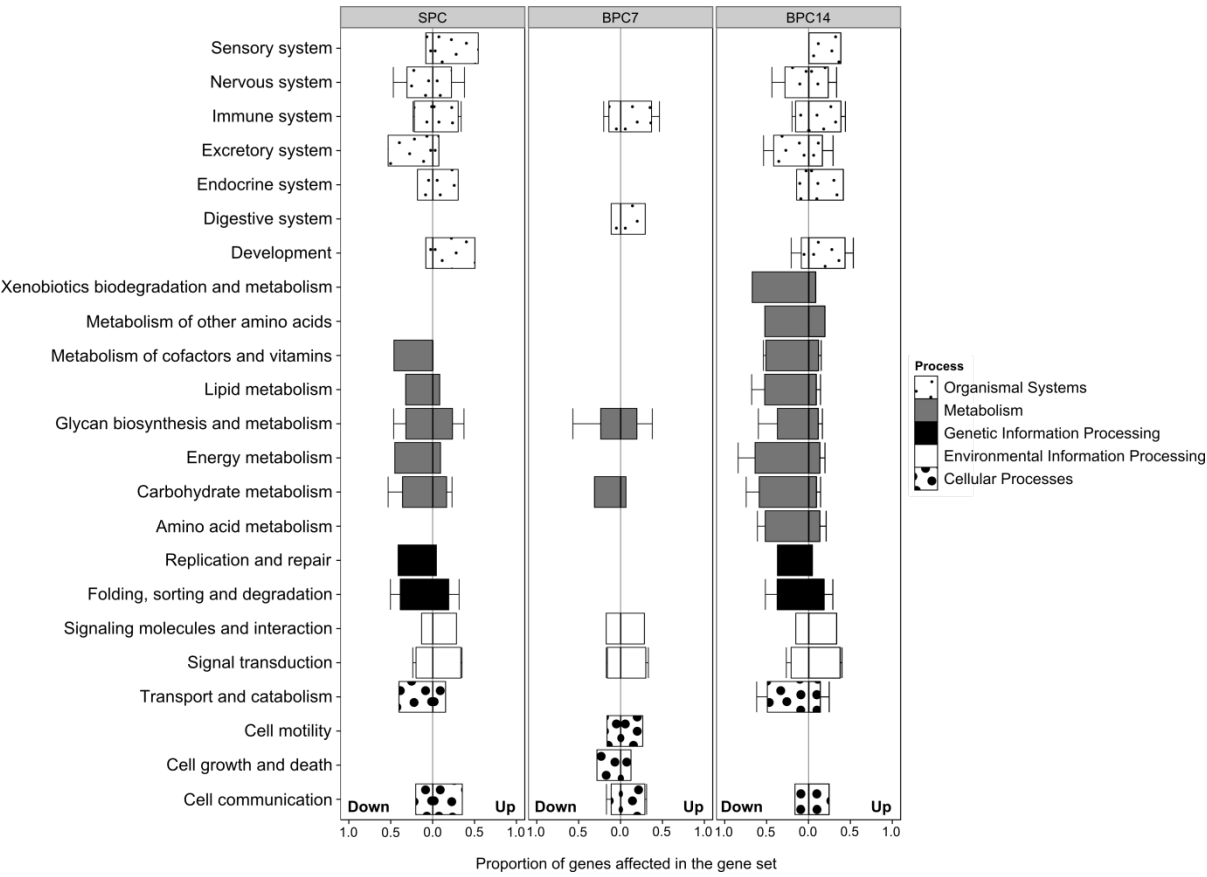
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34 Fig.4.
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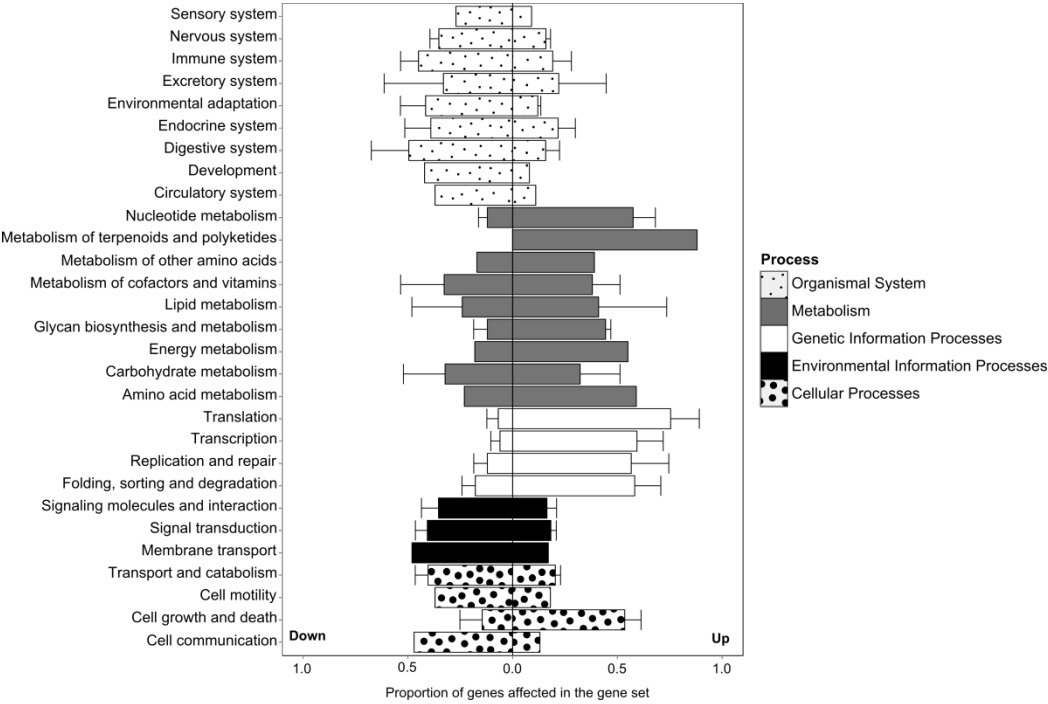


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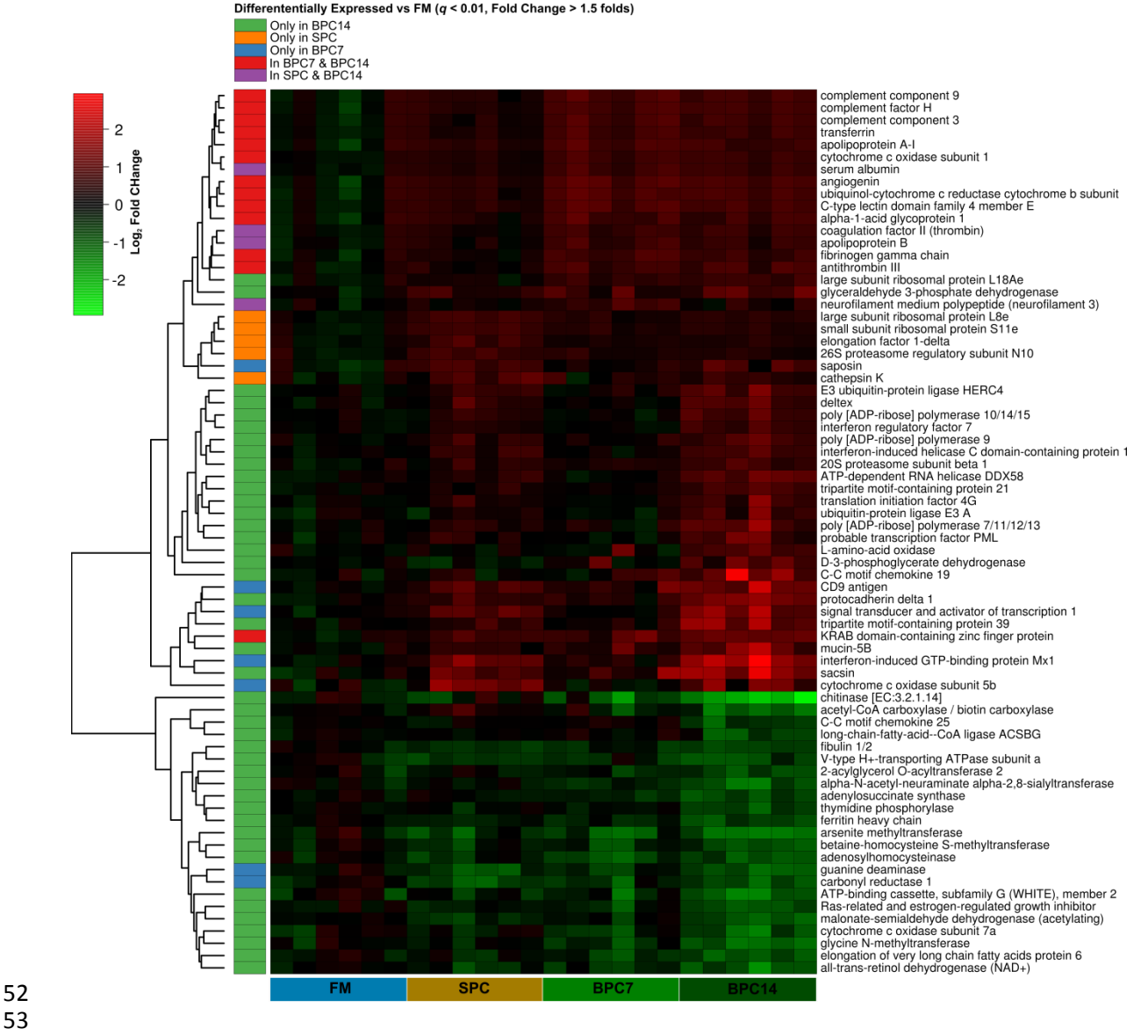
39 Fig.5.
40



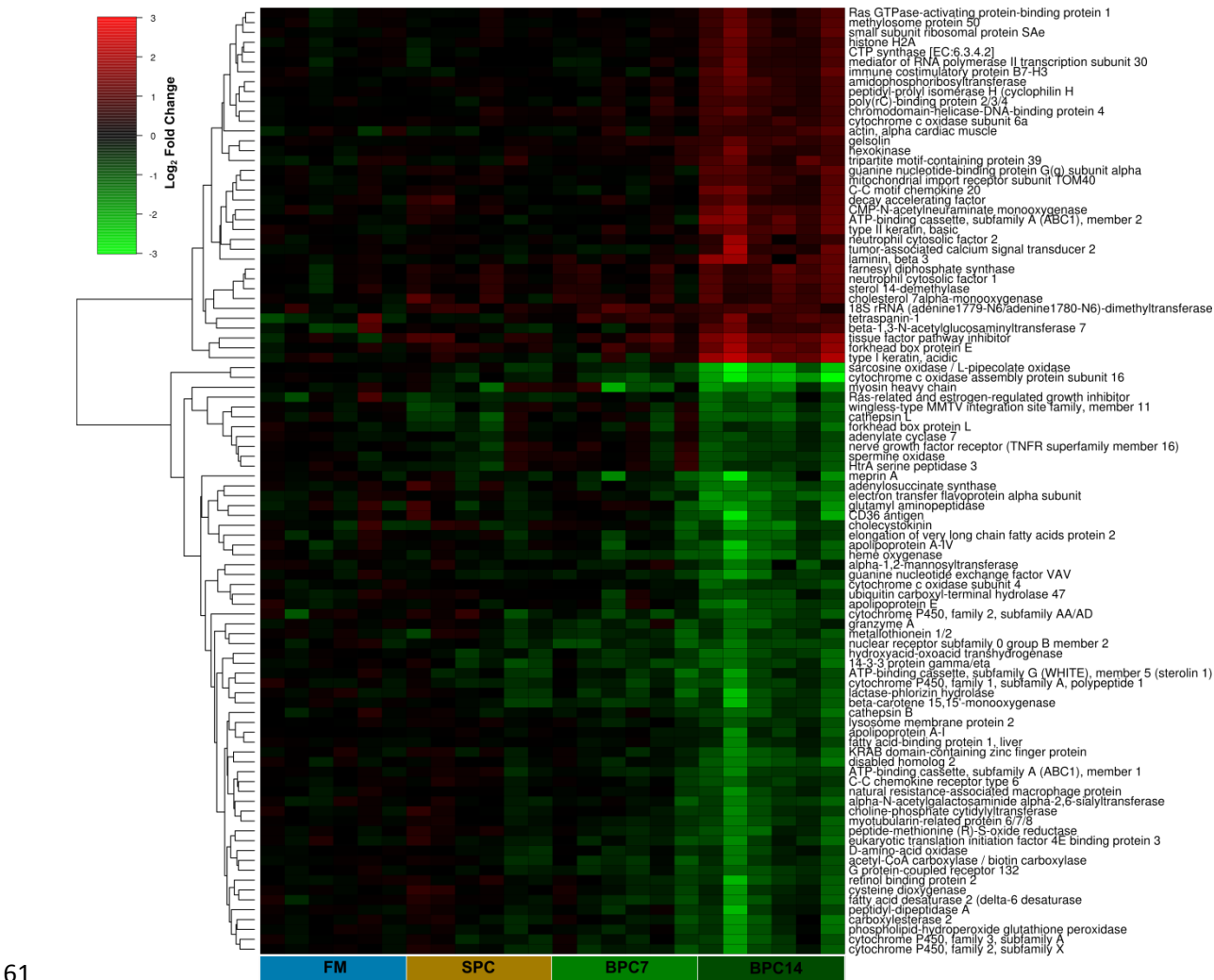
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46 **Supplementary Figure 1.** Heatmap plotting the log₂ transformed expression (relatively to FM)
 47 in a selection of differentially expressed genes in liver. Genes plotted were selected for being
 48 differentially expressed in at least one of the contrast SPC vs FM, BPI7 vs FM, BPI14 vs FM
 49 with absolute fold change > 1.5 and $q < 0.01$ ($q = p$ value corrected for false discovery rate). For
 50 each treatment all replicates are plotted. The heatmap was generated using the package gplots
 51 (Warnes et al., 2013).



54 **Supplementary Figure 2.** Heatmap plotting the \log_2 transformed expression (relatively to FM)
 55 in a selection of differentially expressed genes in pyloric caeca. Genes plotted were selected for
 56 being differentially expressed in at least one of the contrast SPC vs FM, BPI7 vs FM, BPI14 vs
 57 FM with absolute fold change > 1.5 and $q < 0.01$ ($q = p$ value corrected for false discovery rate),
 58 however no genes were differentially expressed in SPC vs FM and BPI7 vs FM under these
 59 selection cutoff. For each treatment all replicates are plotted. The heatmap was generated using
 60 the package gplots (Warnes et al., 2013).



Feed Intake

Fit: lmer(formula = FeedConsumed ~ Treatment + (1 | Date) + (1 | Treatment:Pen), data = FeedIntake)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	0.0656	0.02576	2.546	0.0532	.
BPI7 - FM == 0	0.10313	0.02576	4.003	<0.001	***
BPI14 - FM == 0	-0.17411	0.02576	-6.758	<0.001	***
BPI7 - SPC == 0	0.03753	0.02576	1.457	0.4639	
BPI14 - SPC == 0	-0.23971	0.02576	-9.304	<0.001	***
BPI14 - BPI7 == 0	-0.27724	0.02576	-10.761	<0.001	***

FCR

Fit: lmer(formula = FCR ~ Treatment + (1 | Treatment:Pen), data = growthdata)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	-0.007116	0.025451	-0.28	0.9924	
BPI7 - FM == 0	0.033396	0.025451	1.312	0.5551	
BPI14 - FM == 0	0.10509	0.025451	4.129	<0.001	***
BPI7 - SPC == 0	0.040512	0.025451	1.592	0.3833	
BPI14 - SPC == 0	0.112206	0.025451	4.409	<0.001	***
BPI14 - BPI7 == 0	0.071694	0.025451	2.817	0.0248	*

Weight

Fit: lmer(formula = FINweights ~ Treatment + INweights + (1 | Treatment:Pen), data = growthdata)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	41.598	10.541	3.946	<0.001	***
BPI7 - FM == 0	24.877	8.91	2.792	0.0268	*
BPI14 - FM == 0	-124.408	8.94	-13.916	<0.001	***
BPI7 - SPC == 0	-16.721	10.651	-1.57	0.394	
BPI14 - SPC == 0	-166.006	10.452	-15.883	<0.001	***
BPI14 - BPI7 == 0	-149.285	8.941	-16.697	<0.001	***

Length

Fit: lmer(formula = lengths ~ Treatment + (1 | Treatment:Pen), data = growthdata)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	0.3267	0.2933	1.114	0.6811	
BPI7 - FM == 0	0.44	0.2933	1.5	0.4374	
BPI14 - FM == 0	-1.0467	0.2933	-3.568	0.0019	**
BPI7 - SPC == 0	0.1133	0.2933	0.386	0.9804	
BPI14 - SPC == 0	-1.3733	0.2933	-4.682	<0.001	***
BPI14 - BPI7 == 0	-1.4867	0.2933	-5.069	<0.001	***

K factor

Fit: lmer(formula = Kfactor ~ Treatment + (1 | Treatment:Pen), data = growthdata)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	0.02193	0.02546	0.861	0.82478	
BPI7 - FM == 0	-0.01828	0.02546	-0.718	0.889942	
BPI14 - FM == 0	-0.12241	0.02546	-4.808	< 1e-04	***
BPI7 - SPC == 0	-0.04021	0.02546	-1.579	0.390382	
BPI14 - SPC == 0	-0.14434	0.02546	-5.669	< 1e-04	***
BPI14 - BPI7 == 0	-0.10413	0.02546	-4.09	0.000243	***

HSI

Fit: lmer(formula = HSI ~ Treatment + (1 | Treatment:Pen), data = vsihsi)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	0.10103	0.06598	1.531	0.41869	
BPI7 - FM == 0	0.21718	0.06598	3.292	0.00533	**
BPI14 - FM == 0	0.32385	0.06598	4.908	< 0.001	***
BPI7 - SPC == 0	0.11615	0.06598	1.761	0.29259	
BPI14 - SPC == 0	0.22282	0.06598	3.377	0.00403	**
BPI14 - BPI7 == 0	0.10667	0.06598	1.617	0.36908	

VSI

Fit: lmer(formula = VSI ~ Treatment + (1 | Treatment:Pen), data = vsihsi)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	-0.1964	0.4273	-0.46	0.968	
BPI7 - FM == 0	0.3372	0.4273	0.789	0.859	
BPI14 - FM == 0	2.4926	0.4273	5.834	<1e-04	***
BPI7 - SPC == 0	0.5336	0.4273	1.249	0.596	
BPI14 - SPC == 0	2.689	0.4273	6.293	<1e-04	***
BPI14 - BPI7 == 0	2.1554	0.4273	5.045	<1e-04	***

Oil Content

Fit: lmer(formula = Oil ~ Treatment + (1 | Pen), data = Mopa)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	-0.008333	0.604543	-0.014	1	
BPI7 - FM == 0	0.503333	0.604543	0.833	0.839	
BPI14 - FM == 0	-3.753333	0.604543	-6.209	<1e-05	***
BPI7 - SPC == 0	0.511667	0.604543	0.846	0.832	
BPI14 - SPC == 0	-3.745	0.604543	-6.195	<1e-05	***
BPI14 - BPI7 == 0	-4.256667	0.604543	-7.041	<1e-05	***

Moisture Content

Fit: lmer(formula = Moisture ~ Treatment + (1 | Pen), data = Mopa)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)	
SPC - FM == 0	-0.3567	0.6231	-0.572	0.94	
BPI7 - FM == 0	-0.56	0.6231	-0.899	0.805	
BPI14 - FM == 0	3.0183	0.6231	4.844	<1e-05	***
BPI7 - SPC == 0	-0.2033	0.6231	-0.326	0.988	
BPI14 - SPC == 0	3.375	0.6231	5.417	<1e-05	***
BPI14 - BPI7 == 0	3.5783	0.6231	5.743	<1e-05	***

Ash Content

Fit: lmer(formula = Ash ~ Treatment + (1 | Pen), data = Mopa)

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)
SPC - FM == 0	-0.035	0.08992	-0.389	0.98
BPI7 - FM == 0	-0.01333	0.08992	-0.148	0.999
BPI14 - FM == 0	-0.13833	0.08992	-1.538	0.414
BPI7 - SPC == 0	0.02167	0.08992	0.241	0.995
BPI14 - SPC == 0	-0.10333	0.08992	-1.149	0.659
BPI14 - BPI7 == 0	-0.125	0.08992	-1.39	0.505

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Adjusted p values reported -- single-step method)

65

66

Supplementary Table 2. Gene sets (pathways) affected by diet in liver.

Diet	Pathway	Number of Genes	Proportion Up	Proportion Down	FDR	Biological Process	Class
SPC	ko04520 Adherens junction	54	0.35	0.20	0.046	Cellular Processes	Cell communication
SPC	ko04146 Peroxisome	62	0.15	0.40	0.049	Cellular Processes	Transport and catabolism
SPC	ko04390 Hippo signaling pathway	79	0.33	0.19	0.002	Environmental Information Processing	Signal transduction
SPC	ko04310 Wnt signaling pathway	72	0.36	0.19	0.005	Environmental Information Processing	Signal transduction
SPC	ko04630 Jak-STAT signaling pathway	67	0.33	0.16	0.010	Environmental Information Processing	Signal transduction
SPC	ko04370 VEGF signaling pathway	30	0.33	0.17	0.012	Environmental Information Processing	Signal transduction
SPC	ko04350 TGF-beta signaling pathway	50	0.32	0.20	0.034	Environmental Information Processing	Signal transduction
SPC	ko04066 HIF-1 signaling pathway	60	0.32	0.27	0.043	Environmental Information Processing	Signal transduction
SPC	ko04060 Cytokine-cytokine receptor interaction	115	0.28	0.13	0.020	Environmental Information Processing	Signaling molecules and interaction
SPC	ko03018 RNA degradation	53	0.21	0.42	0.011	Genetic Information Processing	Folding, sorting and degradation
SPC	ko04130 SNARE interactions in vesicular transport	27	0.04	0.33	0.012	Genetic Information Processing	Folding, sorting and degradation
SPC	ko04120 Ubiquitin mediated proteolysis	102	0.14	0.26	0.012	Genetic Information Processing	Folding, sorting and degradation
SPC	ko03060 Protein export	19	0.36	0.53	0.016	Genetic Information Processing	Folding, sorting and degradation
SPC	ko03410 Base excision repair	27	0.04	0.41	0.010	Genetic Information Processing	Replication and repair
SPC	ko00052 Galactose metabolism	17	0.24	0.18	0.020	Metabolism	Carbohydrate metabolism
SPC	ko00520 Amino sugar and nucleotide sugar metabolism	36	0.19	0.36	0.022	Metabolism	Carbohydrate metabolism
SPC	ko00630 Glyoxylate and dicarboxylate metabolism	20	0.10	0.30	0.031	Metabolism	Carbohydrate metabolism
SPC	ko00650 Butanoate metabolism	17	0.12	0.59	0.031	Metabolism	Carbohydrate metabolism
SPC	ko00720 Carbon fixation pathways in prokaryotes	11	0.09	0.45	0.026	Metabolism	Energy metabolism
SPC	ko00563 Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	17	0.06	0.41	0.002	Metabolism	Glycan biosynthesis and metabolism
SPC	ko00514 Other types of O-glycan biosynthesis	19	0.37	0.16	0.015	Metabolism	Glycan biosynthesis and metabolism
SPC	ko00510 N-Glycan biosynthesis	36	0.19	0.47	0.018	Metabolism	Glycan biosynthesis and metabolism
SPC	ko00531 Glycosaminoglycan degradation	13	0.31	0.23	0.028	Metabolism	Glycan biosynthesis and metabolism
SPC	ko00590 Arachidonic acid metabolism	25	0.08	0.32	0.031	Metabolism	Lipid metabolism
SPC	ko00670 One carbon pool by folate	13	0.00	0.46	0.002	Metabolism	Metabolism of cofactors and vitamins
SPC	ko04320 Dorsal-ventral axis formation	12	0.50	0.08	0.002	Organismal Systems	Development
SPC	ko04916 Melanogenesis	44	0.30	0.18	0.010	Organismal Systems	Endocrine system
SPC	ko04966 Collecting duct acid secretion	15	0.07	0.53	0.031	Organismal Systems	Excretory system
SPC	ko04650 Natural killer cell mediated cytotoxicity	49	0.33	0.20	0.032	Organismal Systems	Immune system
SPC	ko04640 Hematopoietic cell lineage	45	0.31	0.22	0.045	Organismal Systems	Immune system
SPC	ko04062 Chemokine signaling pathway	98	0.26	0.23	0.049	Organismal Systems	Immune system
SPC	ko04724 Glutamatergic synapse	64	0.33	0.19	0.019	Organismal Systems	Nervous system
SPC	ko04721 Synaptic vesicle cycle	38	0.11	0.42	0.046	Organismal Systems	Nervous system
SPC	ko04742 Taste transduction	13	0.54	0.08	0.010	Organismal Systems	Sensory system
BP17	ko04540 Gap junction	45	0.27	0.07	0.006	Cellular Processes	Cell communication
BP17	ko04530 Tight junction	61	0.30	0.15	0.019	Cellular Processes	Cell communication
BP17	ko04113 Meiosis - yeast	43	0.12	0.28	0.031	Cellular Processes	Cell growth and death
BP17	ko04810 Regulation of actin cytoskeleton	117	0.26	0.16	0.036	Cellular Processes	Cell motility
BP17	ko04310 Wnt signaling pathway	72	0.29	0.15	0.012	Environmental Information Processing	Signal transduction
BP17	ko04390 Hippo signaling pathway	79	0.27	0.15	0.019	Environmental Information Processing	Signal transduction
BP17	ko04370 VEGF signaling pathway	30	0.33	0.17	0.043	Environmental Information Processing	Signal transduction
BP17	ko04060 Cytokine-cytokine receptor interaction	115	0.28	0.17	0.039	Environmental Information Processing	Signaling molecules and interaction
BP17	ko00520 Amino sugar and nucleotide sugar metabolism	36	0.06	0.31	0.038	Metabolism	Carbohydrate metabolism
BP17	ko00563 Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	17	0.06	0.47	0.006	Metabolism	Glycan biosynthesis and metabolism
BP17	ko00514 Other types of O-glycan biosynthesis	19	0.32	0.00	0.009	Metabolism	Glycan biosynthesis and metabolism
BP17	ko04970 Salivary secretion	38	0.29	0.11	0.038	Organismal Systems	Digestive system
BP17	ko04062 Chemokine signaling pathway	98	0.27	0.15	0.023	Organismal Systems	Immune system
BP17	ko04621 NOD-like receptor signaling pathway	34	0.32	0.09	0.023	Organismal Systems	Immune system
BP17	ko04666 Fc gamma R-mediated phagocytosis	45	0.49	0.22	0.024	Organismal Systems	Immune system
BP17	ko04612 Antigen processing and presentation	34	0.38	0.09	0.030	Organismal Systems	Immune system
BP14	ko04540 Gap junction	45	0.24	0.16	0.014	Cellular Processes	Cell communication
BP14	ko04146 Peroxisome	62	0.06	0.58	0.001	Cellular Processes	Transport and catabolism
BP14	ko04142 Lysosome	89	0.21	0.40	0.028	Cellular Processes	Transport and catabolism
BP14	ko04390 Hippo signaling pathway	79	0.39	0.15	0.001	Environmental Information Processing	Signal transduction
BP14	ko04630 Jak-STAT signaling pathway	67	0.34	0.18	0.001	Environmental Information Processing	Signal transduction
BP14	ko04340 Hedgehog signaling pathway	23	0.39	0.17	0.022	Environmental Information Processing	Signal transduction
BP14	ko04330 Notch signaling pathway	21	0.38	0.29	0.029	Environmental Information Processing	Signal transduction
BP14	ko04310 Wnt signaling pathway	72	0.36	0.24	0.030	Environmental Information Processing	Signal transduction
BP14	ko04060 Cytokine-cytokine receptor interaction	115	0.33	0.15	0.001	Environmental Information Processing	Signaling molecules and interaction
BP14	ko04130 SNARE interactions in vesicular transport	27	0.04	0.44	0.003	Genetic Information Processing	Folding, sorting and degradation
BP14	ko04120 Ubiquitin mediated proteolysis	102	0.26	0.21	0.048	Genetic Information Processing	Folding, sorting and degradation
BP14	ko04141 Protein processing in endoplasmic reticulum	118	0.16	0.31	0.048	Genetic Information Processing	Folding, sorting and degradation
BP14	ko03060 Protein export	19	0.26	0.53	0.048	Genetic Information Processing	Folding, sorting and degradation
BP14	ko03410 Base excision repair	27	0.04	0.37	0.017	Genetic Information Processing	Replication and repair
BP14	ko00280 Valine, leucine and isoleucine degradation	38	0.05	0.63	0.001	Metabolism	Amino acid metabolism
BP14	ko00380 Tryptophan metabolism	30	0.07	0.60	0.003	Metabolism	Amino acid metabolism
BP14	ko00310 Lysine degradation	33	0.21	0.42	0.008	Metabolism	Amino acid metabolism
BP14	ko00250 Alanine, aspartate and glutamate metabolism	24	0.21	0.54	0.009	Metabolism	Amino acid metabolism
BP14	ko00260 Glycine, serine and threonine metabolism	30	0.17	0.50	0.011	Metabolism	Amino acid metabolism
BP14	ko00340 Histidine metabolism	15	0.07	0.40	0.050	Metabolism	Amino acid metabolism
BP14	ko00520 Amino sugar and nucleotide sugar metabolism	36	0.14	0.39	0.001	Metabolism	Carbohydrate metabolism
BP14	ko00620 Pyruvate metabolism	26	0.04	0.77	0.001	Metabolism	Carbohydrate metabolism
BP14	ko00630 Glyoxylate and dicarboxylate metabolism	20	0.05	0.65	0.001	Metabolism	Carbohydrate metabolism
BP14	ko00640 Propanoate metabolism	22	0.09	0.59	0.001	Metabolism	Carbohydrate metabolism
BP14	ko00650 Butanoate metabolism	17	0.06	0.71	0.001	Metabolism	Carbohydrate metabolism
BP14	ko00020 Citrate cycle (TCA cycle)	23	0.04	0.74	0.003	Metabolism	Carbohydrate metabolism
BP14	ko00040 Pentose and glucuronate interconversions	14	0.07	0.50	0.006	Metabolism	Carbohydrate metabolism
BP14	ko00030 Pentose phosphate pathway	18	0.17	0.61	0.010	Metabolism	Carbohydrate metabolism
BP14	ko00500 Starch and sucrose metabolism	22	0.14	0.32	0.010	Metabolism	Carbohydrate metabolism
BP14	ko00720 Carbon fixation pathways in prokaryotes	11	0.09	0.82	0.001	Metabolism	Energy metabolism
BP14	ko00190 Oxidative phosphorylation	105	0.10	0.42	0.002	Metabolism	Energy metabolism
BP14	ko00710 Carbon fixation in photosynthetic organisms	15	0.20	0.67	0.019	Metabolism	Energy metabolism
BP14	ko00563 Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	17	0.06	0.53	0.002	Metabolism	Glycan biosynthesis and metabolism
BP14	ko00510 N-Glycan biosynthesis	36	0.11	0.47	0.005	Metabolism	Glycan biosynthesis and metabolism
BP14	ko00514 Other types of O-glycan biosynthesis	19	0.16	0.11	0.048	Metabolism	Glycan biosynthesis and metabolism
BP14	ko01040 Biosynthesis of unsaturated fatty acids	15	0.13	0.80	0.001	Metabolism	Lipid metabolism
BP14	ko00564 Glycerophospholipid metabolism	48	0.13	0.38	0.002	Metabolism	Lipid metabolism
BP14	ko00062 Fatty acid elongation	18	0.11	0.67	0.003	Metabolism	Lipid metabolism
BP14	ko00140 Steroid hormone biosynthesis	25	0.04	0.44	0.004	Metabolism	Lipid metabolism
BP14	ko00590 Arachidonic acid metabolism	25	0.12	0.40	0.006	Metabolism	Lipid metabolism
BP14	ko00100 Steroid biosynthesis	14	0.00	0.50	0.010	Metabolism	Lipid metabolism
BP14	ko00120 Primary bile acid biosynthesis	13	0.08	0.46	0.032	Metabolism	Lipid metabolism
BP14	ko00860 Porphyrin and chlorophyll metabolism	21	0.10	0.48	0.001	Metabolism	Metabolism of cofactors and vitamins
BP14	ko00670 One carbon pool by folate	13	0.15	0.54	0.002	Metabolism	Metabolism of cofactors and vitamins
BP14	ko00830 Retinol metabolism	22	0.09	0.50	0.003	Metabolism	Metabolism of cofactors and vitamins
BP14	ko00410 beta-Alanine metabolism	21	0.19	0.52	0.015	Metabolism	Metabolism of other amino acids
BP14	ko00980 Metabolism of xenobiotics by cytochrome P450	12	0.08	0.67	0.004	Metabolism	Xenobiotics biodegradation and metabolism
BP14	ko04320 Dorsal-ventral axis formation	12	0.50	0.00	0.003	Organismal Systems	Development
BP14	ko04380 Osteoclast differentiation	78	0.36	0.17	0.012	Organismal Systems	Development
BP14	ko04916 Melanogenesis	44	0.41	0.14	0.007	Organismal Systems	Endocrine system
BP14	ko04964 Proximal tubule bicarbonate reclamation	12	0.25	0.50	0.008	Organismal Systems	Excretory system
BP14	ko04966 Collecting duct acid secretion	15	0.07	0.33	0.032	Organismal Systems	Excretory system
BP14	ko04640 Hematopoietic cell lineage	45	0.31	0.16	0.001	Organismal Systems	Immune system
BP14	ko04622 RIG-I-like receptor signaling pathway	40	0.38	0.13	0.002	Organismal Systems	Immune system
BP14	ko04623 Cytosolic DNA-sensing pathway	36	0.39	0.19	0.002	Organismal Systems	Immune system
BP14	ko04666 Fc gamma R-mediated phagocytosis	45	0.47	0.18	0.004	Organismal Systems	Immune system
BP14	ko04062 Chemokine signaling pathway	98	0.36	0.19	0.004	Organismal Systems	Immune system
BP14	ko04612 Antigen processing and presentation	34	0.44	0.09	0.004	Organismal Systems	Immune system
BP14	ko04621 NOD-like receptor signaling pathway	34	0.32	0.09	0.004	Organismal Systems	Immune system
BP14	ko04620 Toll-like receptor signaling pathway	54	0.35	0.15	0.017	Organismal Systems	Immune system
BP14	ko04650 Natural killer cell mediated cytotoxicity	49	0.41	0.20	0.021	Organismal Systems	Immune system
BP14	ko04724 Glutamatergic synapse	64	0.30	0.17	0.015	Organismal Systems	Nervous system
BP14	ko04721 Synaptic vesicle cycle	38	0.16	0.39	0.027	Organismal Systems	Nervous system
BP14	ko04742 Taste transduction	13	0.38	0.00	0.048	Organismal Systems	Sensory system

Supplementary Table 3. Gene sets (pathways) affected by diet in pyloric caeca.

Diet	Pathway	Number of Genes	Proportion Up	Proportion Down	FDR	Biological Process	Class
BPI14	ko04510 Focal adhesion	120	0.13	0.47	0.003	Cellular Processes	Cell communication
BPI14	ko04113 Meiosis - I	44	0.59	0.07	0.007	Cellular Processes	Cell growth and death
BPI14	ko04111 Cell cycle	57	0.61	0.14	0.008	Cellular Processes	Cell growth and death
BPI14	ko04110 Cell cycle	98	0.48	0.22	0.008	Cellular Processes	Cell growth and death
BPI14	ko04114 Oocyte maturation	66	0.36	0.18	0.024	Cellular Processes	Cell growth and death
BPI14	ko04810 Regulation of actin polymerization	120	0.18	0.37	0.037	Cellular Processes	Cell motility
BPI14	ko04145 Phagosome	79	0.20	0.47	0.031	Cellular Processes	Transport and catabolism
BPI14	ko04142 Lysosome	89	0.18	0.39	0.035	Cellular Processes	Transport and catabolism
BPI14	ko04144 Endocytosis	120	0.23	0.35	0.047	Cellular Processes	Transport and catabolism
BPI14	ko02010 ABC transporters	29	0.17	0.48	0.015	Environmental Information Processing	Membrane transport
BPI14	ko04630 Jak-STAT signaling	65	0.14	0.43	0.005	Environmental Information Processing	Signal transduction
BPI14	ko04012 ErbB signaling	48	0.19	0.42	0.006	Environmental Information Processing	Signal transduction
BPI14	ko04064 NF-kappaB	66	0.15	0.42	0.009	Environmental Information Processing	Signal transduction
BPI14	ko04070 Phosphatidylinositol	33	0.21	0.42	0.020	Environmental Information Processing	Signal transduction
BPI14	ko04515 PI3K-Akt signaling	183	0.21	0.37	0.020	Environmental Information Processing	Signal transduction
BPI14	ko04370 VEGF signaling	31	0.19	0.45	0.026	Environmental Information Processing	Signal transduction
BPI14	ko04390 Hippo signaling	79	0.19	0.42	0.042	Environmental Information Processing	Signal transduction
BPI14	ko04020 Calcium signaling	85	0.16	0.27	0.046	Environmental Information Processing	Signal transduction
BPI14	ko04350 TGF-beta	50	0.20	0.46	0.047	Environmental Information Processing	Signal transduction
BPI14	ko04080 Neurotransmission	151	0.11	0.26	0.006	Environmental Information Processing	Signaling molecules and interaction
BPI14	ko04514 Cell adhesion	72	0.19	0.39	0.020	Environmental Information Processing	Signaling molecules and interaction
BPI14	ko04060 Cytokine-cytokine receptor interaction	111	0.19	0.41	0.029	Environmental Information Processing	Signaling molecules and interaction
BPI14	ko03018 RNA degradation	54	0.63	0.13	0.001	Genetic Information Processing	Folding, sorting and degradation
BPI14	ko03050 Proteasome	41	0.68	0.17	0.010	Genetic Information Processing	Folding, sorting and degradation
BPI14	ko03060 Protein export	21	0.62	0.14	0.010	Genetic Information Processing	Folding, sorting and degradation
BPI14	ko04141 Protein processing in the endoplasmic reticulum	119	0.40	0.27	0.031	Genetic Information Processing	Folding, sorting and degradation
BPI14	ko03410 Base excision repair	29	0.55	0.10	0.001	Genetic Information Processing	Replication and repair
BPI14	ko03420 Nucleotide excision repair	36	0.58	0.08	0.002	Genetic Information Processing	Replication and repair
BPI14	ko03430 Mismatch repair	19	0.79	0.05	0.003	Genetic Information Processing	Replication and repair
BPI14	ko03030 DNA replication	33	0.76	0.12	0.004	Genetic Information Processing	Replication and repair
BPI14	ko03460 Fanconi anemia	41	0.51	0.17	0.005	Genetic Information Processing	Replication and repair
BPI14	ko03440 Homologous recombination	21	0.52	0.24	0.010	Genetic Information Processing	Replication and repair
BPI14	ko03450 Non-homologous end joining	12	0.25	0.08	0.025	Genetic Information Processing	Replication and repair
BPI14	ko03020 RNA polymerase	28	0.68	0.04	0.001	Genetic Information Processing	Transcription
BPI14	ko03022 Basal transcription	33	0.45	0.03	0.001	Genetic Information Processing	Transcription
BPI14	ko03040 Spliceosome	111	0.65	0.11	0.001	Genetic Information Processing	Transcription
BPI14	ko00970 Aminoacyl-tRNA synthetase	28	0.86	0.00	0.001	Genetic Information Processing	Translation
BPI14	ko03008 Ribosome	63	0.87	0.03	0.001	Genetic Information Processing	Translation
BPI14	ko03010 Ribosome	120	0.81	0.09	0.001	Genetic Information Processing	Translation
BPI14	ko03013 RNA translation	114	0.68	0.14	0.001	Genetic Information Processing	Translation
BPI14	ko03015 mRNA surveillance	53	0.55	0.08	0.001	Genetic Information Processing	Translation
BPI14	ko00270 Cysteine metabolism	22	0.59	0.23	0.002	Metabolism	Amino acid metabolism
BPI14	ko00030 Pentose phosphate pathway	18	0.33	0.17	0.008	Metabolism	Carbohydrate metabolism
BPI14	ko00052 Galactose metabolism	17	0.12	0.59	0.010	Metabolism	Carbohydrate metabolism
BPI14	ko00630 Glyoxylate and glyoxal metabolism	20	0.50	0.20	0.023	Metabolism	Carbohydrate metabolism
BPI14	ko00500 Starch and sucrose metabolism	21	0.14	0.48	0.024	Metabolism	Carbohydrate metabolism
BPI14	ko00020 Citrate cycle in the mitochondrion	23	0.52	0.17	0.034	Metabolism	Carbohydrate metabolism
BPI14	ko00720 Carbon fixation in the cytosol	11	0.55	0.18	0.002	Metabolism	Energy metabolism
BPI14	ko00563 Glycosyltransferase	17	0.47	0.06	0.006	Metabolism	Glycan biosynthesis and metabolism
BPI14	ko00510 N-Glycan biosynthesis	38	0.42	0.11	0.026	Metabolism	Glycan biosynthesis and metabolism
BPI14	ko00534 Glycosaminoglycan biosynthesis	16	0.44	0.19	0.043	Metabolism	Glycan biosynthesis and metabolism
BPI14	ko00565 Ether lipid metabolism	17	0.18	0.41	0.007	Metabolism	Lipid metabolism
BPI14	ko00100 Steroid hormone biosynthesis	14	0.64	0.07	0.018	Metabolism	Lipid metabolism
BPI14	ko00760 Nicotinate and nicotinamide metabolism	14	0.43	0.14	0.004	Metabolism	Metabolism of cofactors and vitamins
BPI14	ko00830 Retinol metabolism	22	0.23	0.55	0.018	Metabolism	Metabolism of cofactors and vitamins
BPI14	ko00860 Porphyrin and chlorophyll metabolism	21	0.48	0.29	0.036	Metabolism	Metabolism of cofactors and vitamins
BPI14	ko00480 Glutathione metabolism	23	0.39	0.17	0.027	Metabolism	Metabolism of other amino acids
BPI14	ko00900 Terpenoid biosynthesis	16	0.88	0.00	0.001	Metabolism	Metabolism of terpenoids and polyketides
BPI14	ko00240 Pyrimidine metabolism	74	0.65	0.09	0.001	Metabolism	Nucleotide metabolism
BPI14	ko00230 Purine metabolism	111	0.50	0.15	0.002	Metabolism	Nucleotide metabolism
BPI14	ko04270 Vascular smooth muscle contraction	63	0.11	0.37	0.001	Organismal Systems	Circulatory system
BPI14	ko04320 Dorsal-ventral axis	12	0.08	0.42	0.001	Organismal Systems	Development
BPI14	ko04975 Fat digestion and absorption	19	0.11	0.74	0.002	Organismal Systems	Digestive system
BPI14	ko04977 Vitamin digestion and absorption	16	0.25	0.50	0.002	Organismal Systems	Digestive system
BPI14	ko04970 Salivary gland development	37	0.11	0.32	0.003	Organismal Systems	Digestive system
BPI14	ko04974 Protein digestion and absorption	43	0.16	0.42	0.010	Organismal Systems	Digestive system
BPI14	ko04920 Adipocyte differentiation	38	0.13	0.42	0.001	Organismal Systems	Endocrine system
BPI14	ko04916 Melanogenesis	46	0.15	0.30	0.003	Organismal Systems	Endocrine system
BPI14	ko03320 PPAR signaling pathway	44	0.18	0.50	0.007	Organismal Systems	Endocrine system
BPI14	ko04914 Progesterone biosynthesis	51	0.35	0.22	0.032	Organismal Systems	Endocrine system
BPI14	ko04614 Renin-angiotensin system	11	0.27	0.55	0.043	Organismal Systems	Endocrine system
BPI14	ko04915 Estrogen signaling	51	0.22	0.35	0.045	Organismal Systems	Endocrine system
BPI14	ko04710 Circadian rhythm	18	0.11	0.50	0.001	Organismal Systems	Environmental adaptation
BPI14	ko04713 Circadian rhythm	55	0.13	0.33	0.001	Organismal Systems	Environmental adaptation
BPI14	ko04960 Aldosterone biosynthesis	17	0.06	0.53	0.001	Organismal Systems	Excretory system
BPI14	ko04961 Endocrine system	24	0.38	0.13	0.001	Organismal Systems	Excretory system
BPI14	ko04650 Natural killer cell mediated cytotoxicity	50	0.14	0.54	0.001	Organismal Systems	Immune system
BPI14	ko04664 Fc epsilon receptor	31	0.13	0.55	0.001	Organismal Systems	Immune system
BPI14	ko04660 T cell receptor	68	0.18	0.49	0.001	Organismal Systems	Immune system
BPI14	ko04062 Chemokine	99	0.19	0.44	0.003	Organismal Systems	Immune system
BPI14	ko04662 B cell receptor	44	0.16	0.50	0.010	Organismal Systems	Immune system
BPI14	ko04672 Intestinal immune system	26	0.12	0.38	0.015	Organismal Systems	Immune system
BPI14	ko04640 Hematopoietic system	42	0.21	0.43	0.015	Organismal Systems	Immune system
BPI14	ko04621 NOD-like receptor	23	0.15	0.42	0.016	Organismal Systems	Immune system